

## Transcription efficiency of human apolipoprotein A-I promoter varies with naturally occurring A to G transition

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In human, the gene coding for apolipoprotein A-I (apo A-I), a protein of the plasma lipid transport system, is expressed only in the liver and the intestine. A naturally occurring A to G substitution in the promoter at position -78 was shown to be associated with high density lipoproteins (HDL) in females. We have studied the effect of this mutation on promoter activity using various lengths of promoter sequences and the CAT reporter gene system. Transient expression studies after introduction of these constructs into Hep 3B cells revealed that in the region spanning -330 to +1 of the promoter an A to G substitution increases the activity approximately twofold. On the other hand, when further upstream region (-1469 to +397) is also included, the promoter activity seems comparable in both alleles. Our results show how minimal sequence variations can affect the *in vitro* analysis of promoter activity.

Apolipoprotein A-I; Promoter; CAT assay; Transcription; Human

### 1. INTRODUCTION

In mammals apolipoprotein A-I (apo A-I) is a single polypeptide chain composed of 243 amino acids. It plays a central structural and metabolic role in high density lipoproteins (HDL). In fact apo A-I is the major protein of HDL and a cofactor for lecithin-cholesterol acyltransferase [1]. In most mammalian species the apo A-I synthesis has been demonstrated predominantly in the liver and intestine [2,5]. Small quantities of apo A-I mRNA have also been found in a variety of mammalian tissues [3,4]. Apo A-I promotes cholesterol efflux from cultured cells [6,7], a process called reverse cholesterol transport. This process is mediated by interaction of HDL with its receptor [8] and may protect against atherosclerosis.

The genes coding for apo A-I and for two other apolipoproteins CIII and AIV are closely linked and tandemly organized within a 15-kb DNA segment [9]. In certain patients with premature coronary artery disease, it has been shown that disruption of the structure of this gene cluster results in altered expression of the apo A-I and apo CIII genes leading to combined apo A-I, apo CIII and HDL deficiency, the major risk factor for accelerated atherosclerosis in these patients [10].

The *cis*- and *trans*-acting elements involved in apo A-I gene transcription and their combinational modulation have been extensively studied [11,12]. The DNA

segment located between nucleotides -256 to -41 region upstream from the transcription start site (+1) of the human apo A-I gene contains regulatory elements which are necessary and sufficient for expression in hepatoma (Hep G2) cells [13]. In the human population we have already reported the distribution of a polymorphism due to an adenine (A) to guanine (G) transition, 78 bp upstream from the transcription start site of the apo A-I gene [14]. The DNA region surrounding the polymorphism is a 51-bp fragment that is G-C rich and contains an inverted repeat composed of two 14/15 bp elements [14]. When G is present instead of A at position -78, the homology and self complementarity of the inverted repeats is disrupted. Both direct and inverted repeat sequences within the 5'-flanking regions are involved in the regulation of gene expression [15,16,22]. We have also reported that the A allele is associated with high HDL-C and apo AI levels in women but not in men [14]. In the present manuscript we describe experiments showing that the sequences flanking the mutation are determinant for the differential promoter activity *in vitro* of the apo A-I-G and apo A-I-A alleles.

### 2. MATERIALS AND METHODS

#### 2.1. Cell culture

Hep 3B (human hepatoma) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 µg/ml gentamycin and 2 mM glutamine.

#### 2.2. Construction of CAT fusion genes

For the construction of pTZ190CAT, the CAT gene was excised from pSV2CAT by digesting with *Hind*III and *Bam*HI enzymes and

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cloned into the respective site of pTZ19 (Pharmacia). The A and G variants of the apo A-I promoter were obtained by PCR amplification of the genomic DNA.

Primer FA1.1: 5'-TTAAGCTTCTTCTAGAACACAATGGCAA-CT3' (contains *Hind*III and *Xba*I sites) 5' end at -330 bp.

Primer RA1.1: 5'-TTAAGCTTCTAAGCAGCCAGCTCTTGCA3' (contains a *Hind*III site) 5' end at +1.

The 330-bp fragment obtained was cloned at the respective site in pTZ190 CAT (Fig. 1). The only mutation was confirmed by sequence analysis and the orientation of the insert was confirmed by restriction enzyme analysis.

For the larger fragment of the apo A-I promoter, a genomic subclone [17,18] was used which was digested with *Hind*III and cloned into pTZ190CAT. These (-1469 AI.A CAT, -1469 AI.G CAT) contain the promoter region -1469 to +397 (exon 1, intron 1, exon 2 and 122 nucleotides of intron 2) in the same direction of transcription with the CAT gene (Fig. 1) [13]. These two constructs were digested by *Sma*I (site at -250) and the fragments were exchanged to generate the two new constructs (-1469 AI. AG CAT and -1469 AI.GA CAT). This manipulation was necessary to eliminate any other sequence difference between the A and G alleles. The DNA used for transfection was purified by CsCl gradient centrifugation [19].

### 2.3. DNA transfection and CAT assay

The day before transfection, Hep 3B cells were seeded at a density of  $2 \times 10^5$  cells/60 mm dish and were grown in 5 ml of the medium at 37°C and 5% CO<sub>2</sub> for 20 h. Subsequently, the cells were fed with fresh medium, and 4 h later the DNA (15 µg, calcium phosphate co-precipitate) was added. At 15 to 18 h later the cells were washed once with PBS and supplemented with fresh medium. Forty-eight hours later, the cells were collected by scraping and used for the preparation of protein extracts [19].

Cell protein extracts were made by sonicating washed, pelleted cells in 200 µl of 0.25 M Tris-HCl. For each transfection, half of the extract was incubated for 10 min at 65°C to inactivate endogenous deacetylases and after centrifugation was used for CAT assay [16]. The CAT assay mixture contained 0.25 µCi of [<sup>14</sup>C]chloramphenicol, 250 µM Tris-HCl (pH 7.8), 0.5 mM acetyl-CoA and 80 µl of extract in a final volume of 160 µl. The reaction mixture was incubated at 37°C for 2 h and extracted with 1 ml of ethyl acetate. The ethyl acetate was evaporated and the pellets redissolved in 25 µl of ethyl acetate and analyzed by silica gel TLC (DC-Plastikfolien-Merck) in chloroform/methanol (95:5) for 30 min. The CAT activity was quantitated by cutting out the respective spots and counting. The CAT activity was normalized for efficiency of transfection, which was determined by slot-blot analysis of the cell lysates [20]. The protein concentration of the extract was determined using the Bio-Rad protein assay kit.

## 3. RESULTS AND DISCUSSION

The -330 to +1 AI.A CAT and AI.G CAT plasmids were constructed by PCR amplification of genomic DNA as already described. It was also confirmed by direct sequencing that the A to G substitution at -78 was the only difference between the two constructs. The CAT activity level obtained after transfection of these revealed a reproducibly significant difference between the activity of the two promoters. The CAT activity was determined and normalized for the DNA uptake (by slot-blot hybridization) and for the protein content of the extract. Both DNA uptake and protein content did not show significant variations from experiment to experiment. It was observed that the construct with G at position -78 gives a twofold higher promoter activity (Fig. 2). These results are in contrast to the observation

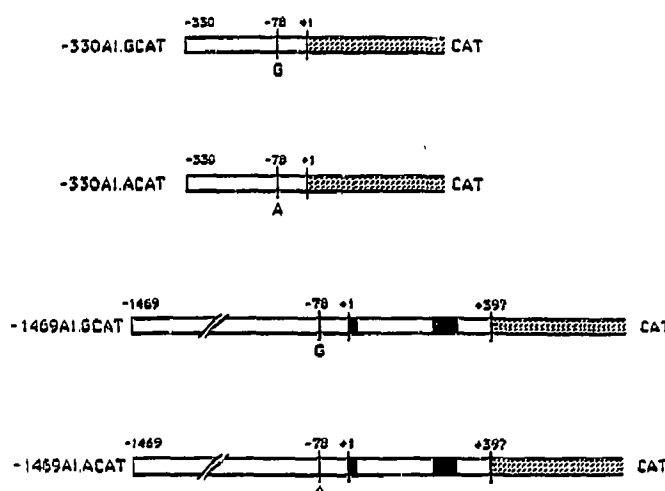


Fig. 1. Structure of the Apo A-I promoter - CAT fusion genes. The upper two are constructed by amplification of genomic DNA and the lower two are subclones of genomic clones.

in vivo, where we found an association of the A allele with high plasma HDL-cholesterol level [14]. The nucleotides -256 to -41 have been previously shown to be an essential *cis*-acting element for the expression of apo A-I in hepatoma cells [13]. Particularly the position -78 is located immediately 3' of the region B that has been shown to be protected (-128 to -77) by nuclear extracts and 5' of a hypersensitive site [21]. The transcription factor interacting with these sequences has not yet been identified [21].

To further explore the interactions involved in the modulation of transcription with both the 'A' and 'G' promoter sequences two constructs were made identical to those previously described [13]. These constructs contain longer upstream promoter region (-1469) and extend at 3' end into exon I, intron I, exon II and 122 nucleotides of intron II of apo A-I fused in the same transcriptional orientation with the bacterial CAT gene (-1469 AI. A CAT and -1469 AI. G. CAT). An identical construct was previously shown to give reliable CAT activity notwithstanding the anomalous inclusion of apo A-I intron sequences [13]. The sequence analysis of -256 to -41 region in these constructs showed them to be identical except for the A to G substitution. No complete sequence information was available for the region -1469 to -250 so to rule out any effect of sequence variations in further upstream region of the promoter (-1469 to -250) on this portion (-250 to +397) two new constructs were made by exchanging the *Sma*I fragment comprising the *Sma*I site at -250 in the apo A-I gene and the *Sma*I site in the polylinker generating the constructs (-1469 AI. AG CAT and -1469 AI. GA CAT). These constructs were used for transient gene expression and the CAT activity of the protein extract of these cells was measured. The results showed that -1469 AI. A CAT is a slightly stronger promoter than

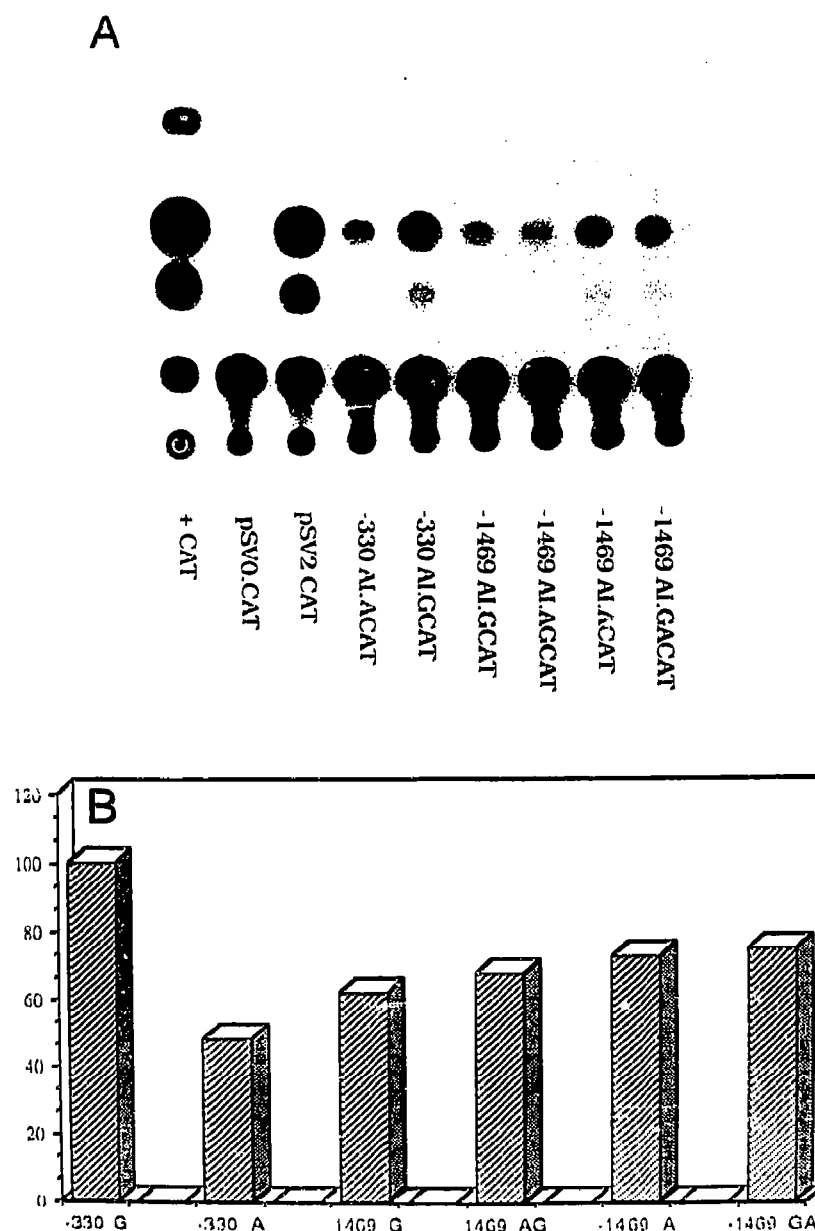


Fig. 2. CAT assay after transfection in Hep 3 B cells. Panel A: autoradiogram of the CAT assay. Lane 1 is the acetylation control using *E. coli* CAT enzyme. The constructs used are shown at the bottom of the autoradiogram. Panel B: schematic representation of CAT assays of panel A. CAT activities are presented in relative values assuming the CAT activity from -330 AI.GCAT to be 100. The difference between the promoter activity of -330 G and -330 A alleles is significant, while that between -1469 G and -1469 A alleles is not. The results shown are averages of at least three independent transfections.

its G counterpart (72.4% vs. 62% of acetylation). Almost identical results were obtained when the two other constructs made after exchanging the *Sma*I fragment were used (Fig. 2, Panel B). The exchange of fragment abolished polymorphic differences but for the A to G substitution, while the difference between -330 'A' and -330 'G' alleles are certain, while the slightly higher -1469 A promoter activity is not significant [14]. Detailed analysis of the nucleotide sequences involved in the Apolipoprotein A-I gene transcription has already been carried out [13,21-23] and some of the transcrip-

tion factors identified [21,23]. The deletion studies have shown previously [13] that the apo A-I promoter activities of constructs including -250 and -2500 flanking regions were comparable. However these studies have been performed with one version of the apo A-I gene. This result would have been different if the A allele was used instead of G allele in the study. In fact we have shown here that the 'A' -330 construct has considerably lower activity than its 'G' counterpart. In the human population there are naturally occurring variants which may have a moderate influence on the rate of transcrip-

tion and may in part explain the wide range of HDL and apo A-I levels found in the population including our previous observation of high HDL in females carrying the 'A' allele [14]. We have shown in this study that a sequence variation may in vitro have a paradoxical effect depending on how much flanking gene sequences are included in the constructs. A complete picture of the interactions and fine modulation of apo A-I gene expression can be obtained only from in vivo experiments using extensive 5'-flanking regions.

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